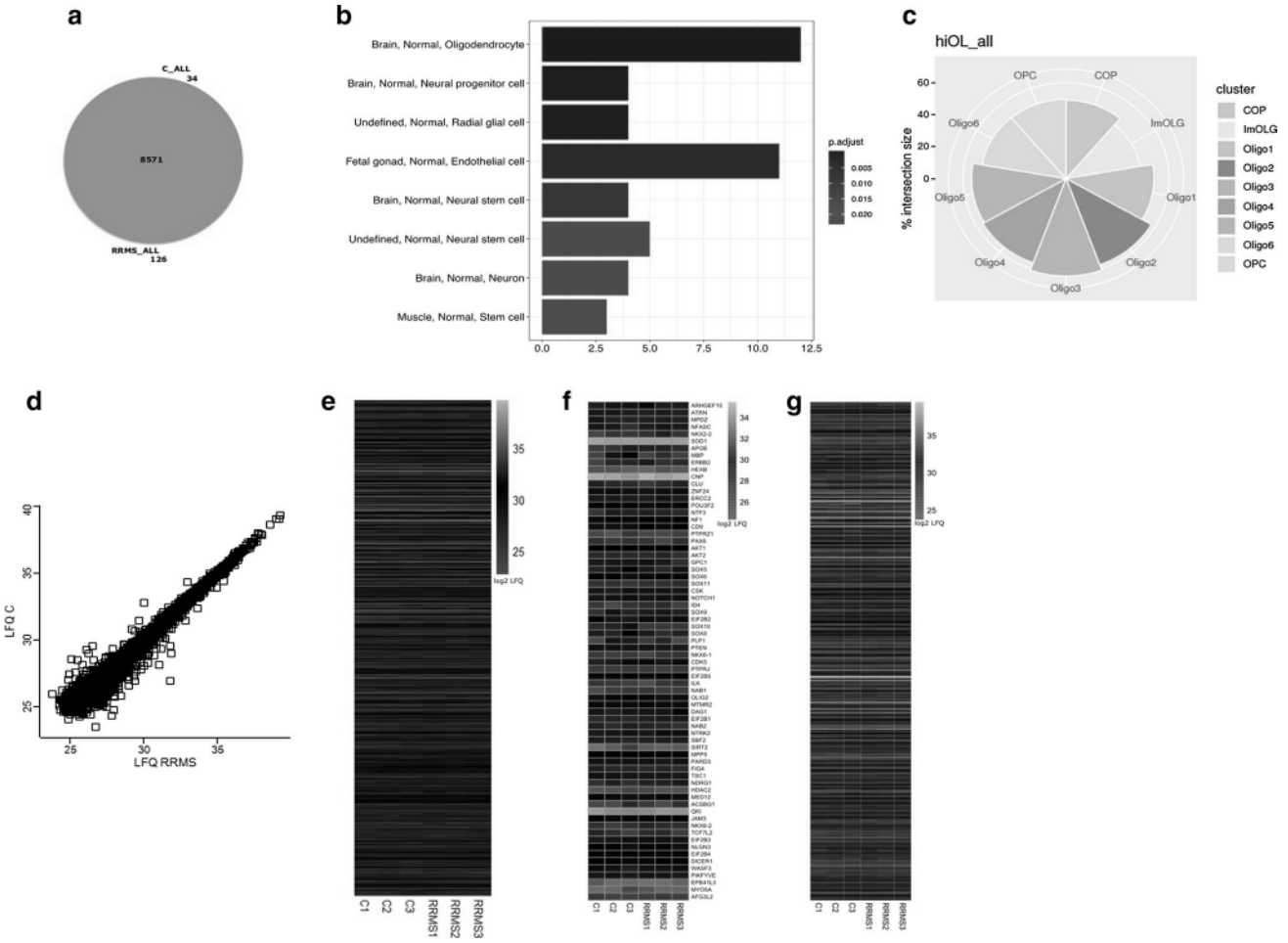


Fig. 3 RRMS and control hiOL display highly similar proteomes. **a** Venn-Diagram showing proteins detected only in pooled control hiOL (C), pooled RRMS hiOL (RRMS) or both indicating that the majority of proteins is shared between both groups. **b** Cell marker enrichment analysis of proteins with mean log2 transformed LFQ intensities > 35 across the whole data set demonstrating that proteins characteristic for oligodendrocytes are significantly enriched in the data set. **c** Polarplot showing the intersection of all proteins with markers characterizing oligodendroglial subclusters identified with single-nucleus RNA sequencing described in Jäkel et al. [23]. Circles indicate percentages of the intersection with distinct clusters. **d** Scatter plot of log2 transformed LFQ intensities of control and RRMS hiOL indicating a strong correlation between the proteomes of RRMS and control hiOL. **e** HeatMap of all identified proteins based on log2 transformed LFQ intensities indicating that protein expression is highly similar between RRMS and control hiOL. **f** HeatMap of log2 transformed LFQ intensities for proteins associated with GO terms "central nervous system myelination", "myelin maintenance", "myelin assembly", "myelination", "oligodendrocyte cell fate specification", "oligodendrocyte development", "oligodendrocyte differentiation", and "positive regulation of oligodendrocyte differentiation" demonstrating presence and similar expression of oligodendroglial proteins in all analyzed samples. **g** HeatMap of log2 transformed LFQ intensities for proteins associated with GO term "immune response" indicating the presence and similar expression levels of proteins connected to the immune system in all analyzed samples. Proteomic analysis was performed in two independent experiments with three RRMS patients and three healthy controls including in total two different NPC clones per patient and healthy control. Statistical significance was determined by Student's *t*-test with permutation-based FDR. See also Supplementary Fig. 5, online resource

To determine the contribution of distinct immune cell types on remyelination in MS, we next analyzed the abundance of $CD68^+$ macrophages/microglia, $CD3^+$ T cells, and $CD20^+$ B cells in active MS lesions since this lesion type is prone to remyelination [53]. Therefore, we investigated biopsies from 32 MS patients and identified lesions based on loss of myelin and relative preservation of axons (Fig. 5c, d). Active lesions are characterized by a diffuse infiltration with numerous $CD68^+$ macrophages/microglia throughout the complete lesion area (Fig. 5e) [30]. As expected, macrophages/microglia were the most abundant immune cell type in active MS lesions (Fig. 5e, h). Additionally, we observed high numbers of T cells (Fig. 5f, i) but very few B cells (Fig. 5g, j). Notably, the number of T cells and B cells but not the number of macrophages/microglia correlated inversely with the extent of remyelination assessed

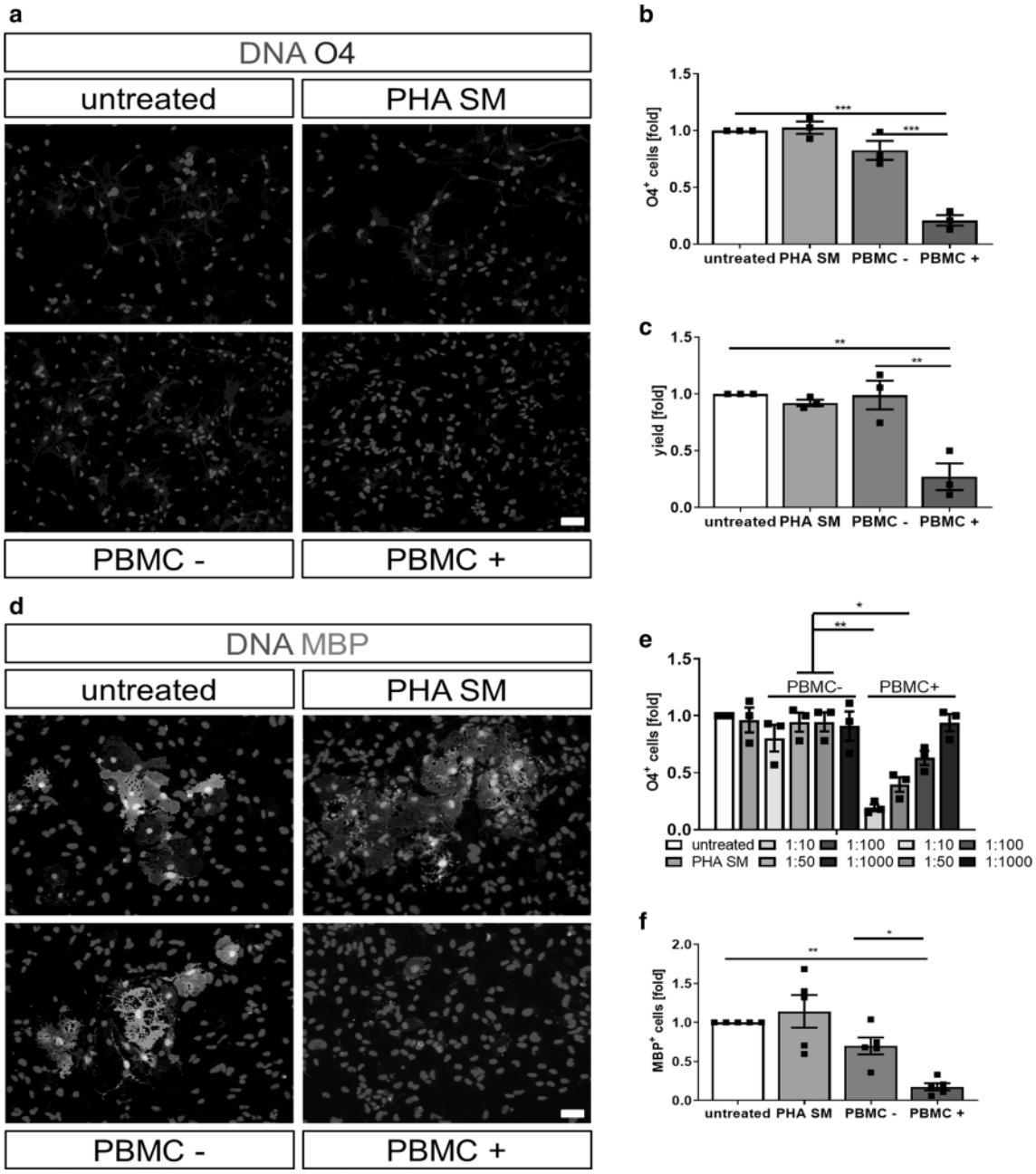


Fig. 4 Supernatants of activated PBMCs significantly impair the differentiation into hiOL. **a** Representative ICC of $O4^+$ (red) hiOL of healthy donors that were either left untreated or treated with PHA-containing stimulation medium (PHA SM) or the supernatants of (non-) activated PBMCs (PBMC–/+) from day 4 until day 21. Flow cytometry-based quantification of $O4^+$ hiOL at day 21 of differentiation indicating a significantly decreased percentage (**b**) and yield (**c**) of $O4^+$ hiOL by PBMC+ ($n=3$). **d** Representative ICC of MBP^+ (green) hiOL at day 35 of differentiation. Untreated cells were sorted by flow cytometry for O4 at day 21 of differentiation and subsequently either left untreated or treated with PHA SM, PBMC–, or PBMC+ until day 35. **e** Flow cytometry-based quantification of $O4^+$ hiOL treated with different dilutions of PBMC supernatants indicating a dose-dependent impaired differentiation into hiOL by PBMC+ at day 21 of differentiation ($n=3$). **f** Quantification of MBP^+ hiOL at day 35 of differentiation by ICC after sorting of untreated $O4^+$ hiOL by flow cytometry at day 21 displaying an impaired differentiation into MBP after treatment with PBMC+ ($n=5$). Data are presented as mean + SEM. Statistical significance was determined by Bonferroni-corrected one-way ANOVA ($*p<0.05$, $**p<0.01$, $***p<0.001$). The relative number of O4/MBP^+ cells present under untreated conditions (**b**, **c**, **e**, **f**) was arbitrarily set to 1 and used to normalize in a pairwise fashion. Scale bars: 50 µm; DAPI was used to counterstain the nuclei. See also Supplementary Fig. 6–9, online resource.

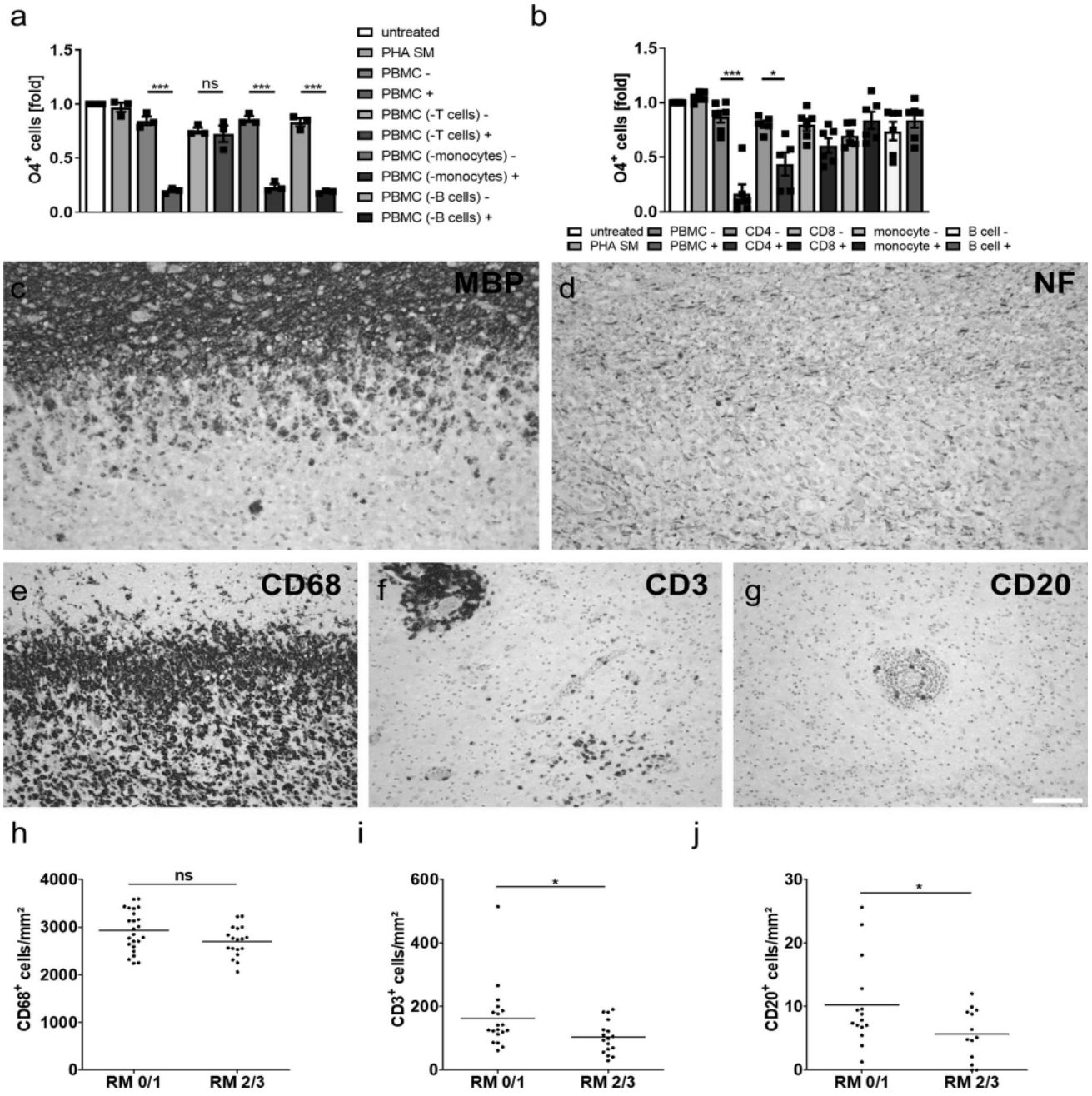


Fig. 5 T cells mediate the impaired oligodendroglial differentiation and correlate negatively with remyelination in MS patients. **a** Flow cytometry-based quantification of $O4^+$ hiOL at day 21 of differentiation. Cells were either left untreated or treated with PHA SM, PBMC−/+, supernatants of (non-) activated monocyte-depleted PBMCs (PBMC (-monocytes)−/+), supernatants of (non-) activated B cell-depleted PBMCs (PBMC (-B-cells)−/+), or supernatants of (non-) activated T cell-depleted PBMCs (PBMC (-T-cells)−/+). Depletion of T cells completely restores differentiation ability of hiOL ($n=3$). **b** Flow cytometry-based quantification of $O4^+$ hiOL at day 21 of differentiation. Cells were either left untreated or treated with PHA SM, PBMC−/+, supernatants of (non-) activated $CD4^+$ T cells (CD4−/+), supernatants of (non-) activated $CD8^+$ T cells (CD8−/+), supernatants of (non-) activated $CD14^+$ monocytes (CD14−/+), or supernatants of (non-) activated $CD19^+$ B cells (CD19−/+). CD4+ significantly inhibit the differentiation of hiOL ($n=6$). Representative IHC of an active MS lesion showing demyelination and relative preservation of axons indicated by MBP (**c**) and neurofilament (NF) (**d**) staining. Infiltration of numerous $CD68^+$ macrophages/microglia (**e**) characterizes active MS lesions. Additionally, $CD3^+$ T cells (**f**) and $CD20^+$ B cells (**g**) are present within the lesions. Analysis of the extent of remyelination (RM) in association with number of $CD68^+$ macrophages/microglia (**h**) ($n=42$), $CD3^+$ T cells (**i**) ($n=36$), and $CD20^+$ B cells (**j**) ($n=28$) demonstrating a correlation between impaired remyelination (RM 0/1) and increased numbers of T cells and B cells but not macrophages/microglia. Data are presented as mean + SEM (**a**, **b**). Statistical significance was determined by Bonferroni-corrected one-way ANOVA (**a**, **b**) or Student's *t* test (**h–j**) (*ns* not significant, $*p<0.05$, $**p<0.01$, $***p<0.001$). The relative number of $O4^+$ cells (**a**, **b**) present under untreated conditions was arbitrarily set to 1 and used to normalize in a pairwise fashion. Scale bar: 100 µm. See also Supplementary Fig. 10, online resource

using a semiquantitative score [18] (Fig. 5h–j). In summary, these in vitro and in vivo data suggest a significant contribution of T cells to impaired remyelination in the CNS.

IFNγ secreted by PBMCs impairs differentiation of hiOL

To elucidate which factors in PBMC+ are responsible for the impaired hiOL differentiation, we heat-inactivated PBMC+. We observed restored differentiation into $O4^+$ hiOL suggesting that the impaired differentiation of hiOL is caused by heat-susceptible factors, such as secreted proteins (Fig. 6a).

Since PBMCs and especially T cells produce high levels of proinflammatory cytokines such as TNFα and IFNγ (Supplementary Fig. 11a, b, online resource), we determined the effects of these two cytokines on oligodendroglial differentiation by exposing differentiating hiOL to recombinant TNFα and IFNγ either alone or in combination. Whereas neither the application of TNFα nor IFNγ alone affected hiOL differentiation, combined application of both cytokines led to a significantly impaired differentiation into hiOL (Fig. 6b). Neutralizing TNFα and IFNγ antibodies resulted in a reduction of TNFα and IFNγ levels in PBMC+ compared to IgG controls (Supplementary Fig. 11c, d, online resource). Neutralizing IFNγ either alone or in addition to TNFα restored the differentiation into $O4^+$ hiOL, whereas blocking of TNFα alone had no effect on impaired hiOL differentiation induced by PBMC+ (Fig. 6c, d). Moreover, yields of $O4^+$ cells were significantly increased after blocking IFNγ or combination of IFNγ and TNFα (Fig. 6e). To determine whether T cells are responsible for IFNγ secretion in PBMC supernatants, we analyzed IFNγ levels in

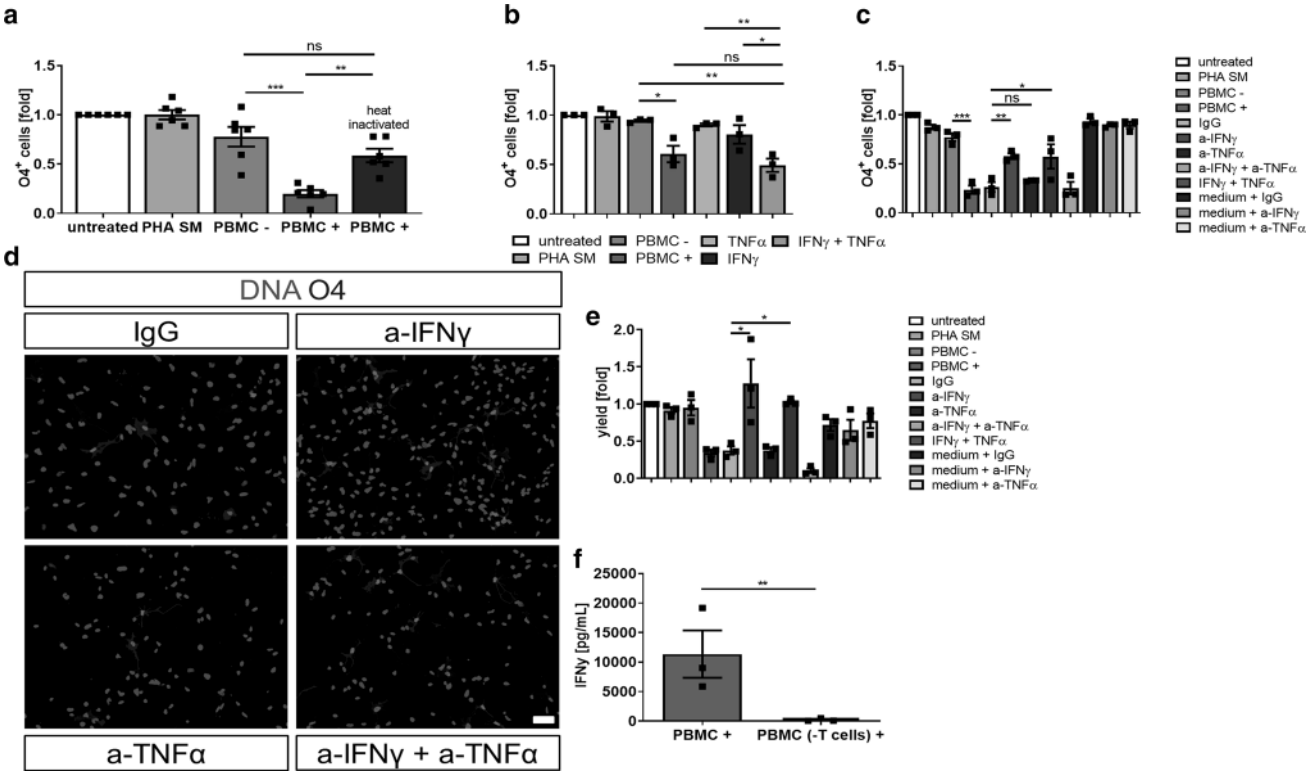
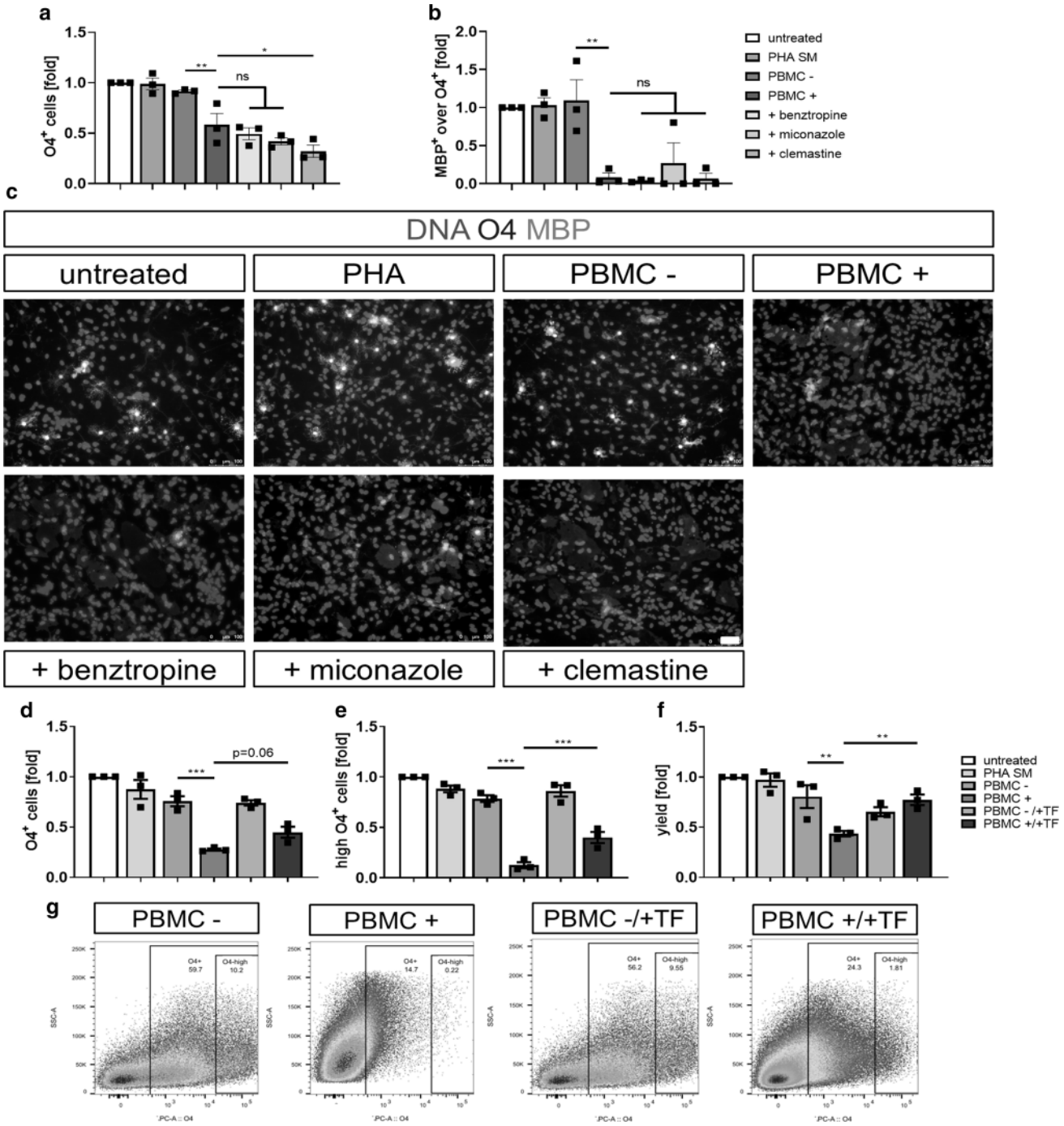


Fig. 6 Neutralization of IFNγ but not TNFα restores the differentiation into hiOL. **a** Flow cytometry-based quantification of $O4^+$ hiOL at day 21 of differentiation. Cells were either left untreated or treated with PHA SM, PBMC−/+, or heat-inactivated PBMC+. Heat inactivation of PBMC+ restores the differentiation into $O4^+$ hiOL ($n=6$). **b** Flow cytometry-based quantification of $O4^+$ hiOL at day 21 of differentiation while being treated with IFNγ (400 ng/mL), TNFα (200 ng/mL), or combination of both showing a significantly impaired differentiation into hiOL when a combination of IFNγ and TNFα was applied ($n=3$). Flow cytometry-based quantification of $O4^+$ hiOL at day 21 of differentiation indicating a significantly enhanced percentage (**c**) and yield (**e**) of $O4^+$ hiOL when PBMC+ were treated with a-IFNγ before compared to IgG ($n=3$). **d** Representative ICC of $O4^+$ (red) hiOL that were treated with PBMC+ which were either incubated with IgG, a-IFNγ, a-TNFα, or a-TNFα + a-IFNγ beforehand. Supernatants were applied from day 4 until day 21 and staining was performed at day 21. **f** ELISA-based analysis of IFNγ in PBMC+ and PBMC (-T cells)+ showing significantly reduced concentrations of IFNγ after depletion of T cells from PBMC+ ($n=3$). Data are presented as mean + SEM. Statistical significance was determined by Bonferroni-corrected one-way ANOVA (**a–c**, **e**) or two-tailed Student's *t* test (**f**) (*ns* not significant, $*p<0.05$, $**p<0.01$, $***p<0.001$). The relative number of $O4^+$ cells (**a–c**, **e**) present under untreated conditions was arbitrarily set to 1 and used to normalize in a pairwise fashion. Scale bar: 50 µm. DAPI was used to counterstain the nuclei. See also Supplementary Figs. 11, 12, online resource



PBMC+ and T cell-depleted PBMC+ (PBMC (−T cells)+) of three different donors. Whereas PBMC+ contained large amounts of IFNγ, IFNγ secretion was significantly reduced in PBMC (−T cells)+ (Fig. 6f) indicating that T cells are essential for IFNγ secretion in PBMCs.

As it was recently described that IFNγ induces the expression of immune markers on rodent oligodendrocytes which suggests a putative role for oligodendrocytes in modulating immune responses [15, 27], we next analyzed whether this also applies to human oligodendrocytes. To this end, we treated differentiating hiOL with PBMC+, PBMC+ in which IFNγ was neutralized, recombinant IFNγ and the combination of recombinant IFNγ and TNFα. We found that PBMC+ as well as IFNγ and TNFα induced the expression of MHC-I, MHC-II and immune adhesion molecules on hiOL on a transcriptional (Supplementary Fig. 12a–e, online resource) and translational level (Supplementary Fig. 12f–j, online resource). Notably, neutralization of IFNγ from PBMC+ reduced enhanced transcript and protein levels. Combined, these data indicate the expression of immune

◂**Fig. 7** Immunomodulatory treatment of PBMCs can partly restore impaired hiOL differentiation induced by supernatants of activated PBMCs. **a** Flow-cytometry based quantification of $O4^+$ hiOL at day 21 of differentiation demonstrating that differentiation-promoting drugs benztropine, miconazole, and clemastine are not able to restore hiOL differentiation under application of PBMC+ ($n=3$). **b** Quantification of MBP^+ over $O4^+$ hiOL at day 35 of differentiation by ICC after sorting of untreated $O4^+$ hiOL by flow cytometry at day 21 displaying that differentiation-promoting drugs benztropine, miconazole, and clemastine are not able to restore impaired hiOL differentiation induced by PBMC+ ($n=3$). **c** Representative ICC of MBP^+ (green) over $O4^+$ (red) hiOL at day 35 of differentiation. Untreated cells were sorted by flow cytometry for O4 at day 21 of differentiation and subsequently either left untreated or treated with PHA SM, PBMC−/+, or PBMC+ plus differentiation-promoting drugs until day 35. Flow cytometry-based quantification of $O4^+$ hiOL (**d**, **f**) or high-$O4^+$ hiOL (**e**) at day 21 of differentiation. Percentage of high-$O4^+$ hiOL and yield of $O4^+$ hiOL are significantly enhanced when PBMCs from three different donors were treated with TF prior to collection of supernatants, while percentage of total $O4^+$ cells shows a trend to increased differentiation ($n=3$). **g** Representative flow cytometry-plots at day 21 of differentiation demonstrating the distribution of $O4^+$ and the subgroup high-$O4^+$ cells after addition of PBMC−, PBMC+, PBMC−/+TF, or PBMC+/+TF to differentiating hiOL. Data are presented as mean + SEM. Statistical significance was determined by Bonferroni-corrected one-way ANOVA (*ns* not significant, $*p<0.05$, $**p<0.01$, $***p<0.001$). The relative number of $O4^+$/MBP^+ over $O4^+$ cells (**a**, **b**, **d–f**) present under untreated conditions was arbitrarily set to 1 and used to normalize in pairwise fashion. Scale bar: 50 µm. DAPI was used to counterstain the nuclei. See also Supplementary Fig. 13, online resource

markers by hiOL in an inflammatory environment which is at least partly mediated by secreted IFNγ.

Immunomodulatory treatment of PBMC partly restores impaired hiOL differentiation mediated by PBMC+

Next, to determine whether impaired hiOL differentiation after application of PBMC+ can be restored by addition of drugs which have been shown to promote oligodendroglial differentiation [12, 14, 41, 42], we applied benztropine, miconazole, and clemastine simultaneously with PBMC+ to differentiating hiOL. Different concentrations of miconazole and benztropine have been shown to promote early and terminal hiOL differentiation in a previous study from our group and we used the most effective concentration identified in this study for our current experiments [14]. We additionally included clemastine although we did not observe a significantly enhanced hiOL differentiation previously [14]. However, it promoted oligodenroglial differentiation in other studies and has been used in clinical trials [19, 41]. Furthermore, the molecular mechanisms required to promote endogenous oligodendroglial differentiation may differ from the mechanisms that promote oligodendroglial differentiation in an inflammatory context. However, none of these drugs was able to rescue impaired early or terminal oligodendroglial differentiation induced by PBMC+ (Fig. 7a–c).

To elucidate whether instead an immunomodulatory treatment of PBMCs before collection of supernatants can restore the differentiation of hiOL, we treated PBMCs from 3 different donors with the immunomodulatory drug teriflunomide (TF) during activation with PHA. Treatment of PBMCs with TF which inhibits the proliferation of immune cells [4] resulted in a significantly reduced but not completely abolished IFNγ secretion during activation (Supplementary Fig. 13, online resource). There was a trend for, but not a significant increase in the percentage of total $O4^+$ cells (Fig. 7d). Furthermore, treatment of PBMCs with TF increased significantly the percentage of strongly $O4^+$ hiOL suggesting an enhanced differentiation in a subpopulation of oligodendrocytes (Fig. 7e and g). Additionally, administration of PBMC+/+TF resulted in significantly enhanced $O4^+$ yields (amount of $O4^+$ cells/amount of initially plated NPCs) (Fig. 7f).

In summary, these data demonstrate that immune cell-derived inflammatory factors such as IFNγ can directly impair oligodendroglial differentiation. Treatment of oligodendrocytes with compounds promoting oligodendroglial differentiation was not able to rescue impaired oligodendroglial differentiation induced by PBMC. However, treatment of PBMCs with the immunomodulatory drug TF before supernatant collection showed a significant effect on the oligodendroglial yield as well as on the differentiation of an oligodendroglial subpopulation suggesting that immunomodulatory drugs might have the potential to increase oligodendroglial differentiation via modulation of the inflammatory environment.

Discussion

Remyelination failure in MS has been attributed to impaired differentiation of OPCs towards mature oligodendrocytes. Prerequisite for successful remyelination in MS is the proliferation and migration of OPCs as well as their differentiation into myelin-forming mature oligodendrocytes. All of these different steps might be impaired in MS lesions; however, impaired oligodendroglial differentiation appears to be a major contributor to remyelination failure in MS [8, 29, 63, 64]. Here, we demonstrate that hiOL from RRMS patients and controls do not differ in their functionality or proteome; however, inflammatory mediators and especially IFNγ, released by T cells, inhibit oligodendroglial differentiation which cannot be restored by the application of oligodendroglial differentiation promoting drugs but can partly be enhanced by imunomodulatory treatment of PBMCs suggesting that the inflammatory environment in MS lesions is a major contributor to impaired remyelination in MS.

During recent years there is a continuing debate over whether primary oligodendroglial pathology might contribute to lesion development and remyelination failure in MS. This hypothesis has been based on the identification of patients with significant oligodendroglial cell death in newly forming MS lesions interpreted as a primary oligodendroglial pathology resulting in secondary inflammation and demyelination [3, 38], the presence of reduced myelin and axonal densities despite lack of inflammation [50], and the description of subgroups of MS patients with either extensive or limited remyelination [46, 47]. Our study is to our best knowledge the first one analyzing in detail functions of human oligodendrocytes required for successful remyelination. Our results suggest that there are no major functional differences between the oligodendrocytes from RRMS patients and controls. This is in line with earlier observations reporting the formation of compact myelin sheaths after transplantation of $O4^+$ iPSC-derived OPCs from four primary progressive MS (PPMS) patients into immunodeficient shiverer mice and the comparable differentiation of iPSC-derived oligodendrocytes from two PPMS patients and two controls into $O4^+$ and MBP^+ oligodendrocytes in vitro [13, 17]. Although the number of patients investigated in these individual studies is low, a caveat immanent to studies using patient-derived iPSCs, they all point in a similar direction, namely that a primary MS-specific oligodendroglial phenotype is not a major contributor to disease pathogenesis in MS. However, a recently published study described increased levels of some cellular senescence markers in NPCs derived from iPSCs from PPMS patients compared to control NPCs. Treatment with rapamycin reduced cellular senescence and restored the capability of NPC supernatants to promote differentiation of rodent oligodendrocytes [43]. These results might indicate that age-specific changes contribute in a disease-specific manner to disease pathology in PPMS.

Recently, using single-nucleus RNA sequencing disease-specific oligodendroglial clusters have been identified in MS patients as well as in experimental autoimmune encephalomyelitis (EAE) mice which serve as an animal model for MS [15, 23], however, the expression of the majority of markers for distinct clusters has not been confirmed on protein level yet. hiOL express all major myelin-associated proteins, such as CNP, PLP, MBP, MAG, MOG etc., confirming the oligodendroglial identity, but also some of the proteins which have been found to be unique or enriched on the transcriptional level in oligodendroglial subclusters identified in MS and control brains, such as PDGFRα, BCAN, SOX6, APOE and CD74 [23]. Additionally, hiOL express paranodal proteins, such as neurofascin and ERMIN. However, the paranodal protein OPALIN is not expressed in hiOL suggesting that the interaction between axons and oligodendrocytes might be required for the upregulation of OPALIN. In their studies Jäckel et al. identified an oligodendroglial subcluster termed immune oligodendroglia (ImOLG) expressing among other markers CD74 and HLA-DRA which were enriched in MS brains [23]. hiOL from RRMS patients and controls express CD74 and HLA-DRA on protein level as well as numerous other immune-related proteins. However, we did not find any significant differences in the expression of these immune-associated proteins between hiOL from RRMS and controls suggesting that an involvement of oligodendrocytes in the immune response is not specific to RRMS hiOL.

Our data suggest that IFNγ, released by T cells, plays a major role in impairing oligodendroglial differentiation in MS but does not induce cell death. IFNγ and TNFα are highly expressed in MS lesions and earlier studies have shown that IFNγ and TNFα are able to induce oligodendroglial cell death [2, 51, 59]. This is in contrast to our data in which exposure to TNFα and IFNγ did not induce oligodendroglial cell death. These discrepancies might be due to species differences, differences in the cytokine concentrations, or distinct developmental stages of oligodendrocytes. Interestingly, recently published studies revealed that treatment with IFNγ enhanced expression of MHC class II proteins on murine OPCs as well as the presentation of antigens suggesting an active role of OPCs during inflammatory processes [15, 27]. Here, we show that an inflammatory environment mimicked by application of PBMC+ or recombinant IFNγ and TNFα also induces the expression of MHC class I and MHC class II proteins as well as several other immune markers on a transcriptional and translational level in hiOL further supporting the notion that oligodendrocytes may play a more active role in the immune response than it has been so far assumed. However, whether and how this contributes to the development and progression of MS remains to be determined.

Consistent with our finding that IFNγ significantly inhibits oligodendroglial differentiation, IFNγ has been reported to reduce developmental myelination and remyelination in the rodent CNS [33, 34]. Next to its detection in CNS tissue sections from patients with MS [65], IFNγ could be attributed to disease progression as its expression has been shown to be increased in the serum of MS patients with ongoing progression compared to patients without progression [25, 26]. Additionally, IFNγ has been connected to impaired differentiation of oligodendrocytes in rodents [1, 9]. Here, we provide the first evidence for an impaired oligodendroglial differentiation in humans caused by IFNγ. However, only the simultaneous application of recombinant IFNγ and TNFα impaired differentiation in hiOL, whereas IFNγ alone did not affect oligodendroglial differentiation. Blocking IFNγ alone, but not TNFα restored differentiation ability of hiOL supporting the major role of IFNγ for impaired oligodendroglial differentiation and suggesting that IFNγ itself is

not sufficient but its presence is essential for immune cell-mediated oligodendroglial differentiation block. In line with this observation, the synergistic effects of IFNγ and TNFα have been reported [2, 7]. Interestingly, treatment with drugs that have been shown to promote oligodendroglial differentiation [12, 14, 41, 42], such as benztropine, miconazole, and clemastine was not able to rescue impaired oligodendroglial differentiation by PBMC+. We used drug concentrations that have been shown before to be efficient in promoting oligodendroglial differentiation; however, we cannot exclude that treatment with other concentrations may have resulted in a different outcome. Treatment of PBMCs prior to supernatant collection with the anti-inflammatory drug TF, which inhibits the proliferation of immune cells [4] and thus reduces the secretion of IFNγ, however, partly restores the differentiation of hiOL. The fact that TF treatment of PBMCs cannot fully restore hiOL differentiation might be explained with the residual IFNγ in PBMC+, as TF does not completely abolish IFNγ secretion in PBMCs. Nevertheless, our findings suggest that drug screenings, which take the inflammatory environment in MS lesions into account, might identify more potent remyelination-promoting compounds.

Our in vitro results demonstrate that IFNγ impairs oligodendroglial differentiation which might be one explanation among others for the negative outcome of a clinical pilot MS trial using IFNγ [45]. Our data could suggest the use of IFNγ inhibiting drugs to promote remyelination. However, blocking IFNγ enhanced disease severity and frequency of relapses in EAE mice [5, 21]. This might be explained by the fact that IFNγ displays dual roles in neuroinflammation. It is not only a proinflammatory cytokine causing damage in the CNS but also displays protective and regulatory roles [44]. Therefore, using neutralizing IFNγ as a treatment option in MS might only be successful in specific disease phases as could be shown in EAE [35]. Nevertheless, blocking of IFNγ induced pathways which inhibit oligodendroglial differentiation might support remyelination and clinical recovery in MS.

In summary, our data suggest that intrinsic oligodendroglial factors do not contribute to impaired remyelination in RRMS while extrinsic factors such as the inflammatory environment impair oligodendroglial differentiation and thereby might affect efficient remyelination. Thus, this data further supports the idea of MS as an immune-mediated rather than a degenerative disorder.

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Author contributions TK initiated the project. TK and LS conceived and designed the study. LS developed and performed the cell culture experiments and analyzed the data, she was initially supported by ME. ML, MH and LK generated and characterized the supernatants from inflammatory cells, KH performed the histological analyses. HD performed the LC–MS/MS and analyzed the data together with CT. MS performed flow cytometry analyses. YKTX quantified the in vitro myelination assays. AR performed karyotype analysis. HRS, JA, JW, LO, FR and GM discussed results and provided critical reagents and comments. LS and TK wrote the manuscript with critical insight and commentary from all co-authors.

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Compliance with ethical standards

Conflict of interest TK and ME have a pending patent application for the generation of human oligodendrocytes.

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